Volume 261, number 1, 171–174 FEBS 08095 February 1990

# Group II phospholipase $A_2$ mRNA synthesis is stimulated by two distinct mechanisms in rat vascular smooth muscle cells

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Received 6 December 1989

Two potent inflammatory mediators, interleukin 1 (IL-1) and tumor necrosis factor (TNF) as well as lipopolysaccharide (LPS) increased group II phospholipase $A_2$ ($PLA_2$) mRNA levels, which resulted in enhanced secretion of the $PLA_2$ enzyme from rat smooth muscle cells. cAMP-elevating agents also stimulated the release of $PLA_2$ and increased the mRNA, but IL-1, TNF and LPS did not affect cAMP levels. Furthermore, the effects of TNF and cAMP-elevating agents were not additive but synergistic. Therefore, we concluded that the level of rat group II $PLA_2$ mRNA is controlled at least by two distinct mechanisms, one involves cAMP and the other is mediated by TNF, IL-1 and LPS. This study also suggests important roles of group II $PLA_2$ in pathogenesis of vascular inflammation.

Interleukin 1; Tumor necrosis factor; Lipopolysaccharide; Phospholipase $A_2$; Smooth muscle cell; Inflammation

## 1. INTRODUCTION

In several inflammatory regions, levels of extracellular $PLA_2$ activity are described to be elevated, which has been thought to play an important role in mediating some inflammatory processes (see for review [1]). In fact, injection of $PLA_2$s into animals has been shown to induce some inflammatory responses [2,3]. Furthermore, two well-known inflammatory mediators, IL-1 [4–7] and TNF [7], have been found to release $PLA_2$ activity from human synovial cells and rabbit and rat chondrocytes. However, the mechanism of the elevation of extracellular $PLA_2$ activity and the type of $PLA_2$ released from the cells have not been clarified yet. $PLA_2$s characterized so far can be classified into two groups, based on their primary structures [8]. Mammalian group I $PLA_2$s exist mainly and abundantly in the pancreas [9–11]. On the other hand, mammalian group II $PLA_2$s have been found in rabbit ascites [12], rat peritoneal exudate [13], human rheumatoid arthritic synovial fluid [14–16], platelets of rabbit [17] and rat [18,19], porcine intestine [20], and rat spleen [21,22]. In this study using rat cultured aortic SMCs, we demonstrated for the first time that there are at least two distinct mechanisms in the activation of group II $PLA_2$ gene expression: one is mediated by inflammatory lymphokines (IL-1 and TNF) as well as LPS, and the other by cAMP-elevating agents.

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*Abbreviations:* IL-1, interleukin 1; TNF, tumor necrosis factor; LPS, lipopolysaccharide; $PLA_2$, phospholipase $A_2$, SMC, smooth muscle cell; IBMX, isobutylmethylxanthine; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol myristate acetate

## 2. MATERIALS AND METHODS

### 2.1. *Isolation and cultivation of smooth muscle cells*

Rat thoracic aortic SMCs were isolated by enzymatic digestion of media of thoracic aorta from male Sprague–Dawley rats as described by Chamley-Campbell et al. [23]. The cells were cultured in DMEM containing 20% fetal calf serum.

### 2.2. *Activation of SMCs*

Confluent SMCs in 90-mm-diameter dishes were washed twice with DMEM and incubated with 8 ml of DMEM containing 0.1 mg/ml bovine serum albumin with or without the agents. At the end of incubation, the supernatant was removed for use in the $PLA_2$ assay and Western blotting.

### 2.3. *$PLA_2$ assay*

Phospholipase activity was measured by the hydrolysis of [$^{14}$C]oleic acid (Amersham)-labeled *Escherichia coli* phospholipids [24]. The assay mixture contained 100 mM Tris, pH 7.4, and 1 mM $CaCl_2$. The medium of the culture was diluted to produce hydrolysis of up to 10% of the substrate. Reaction mixtures were incubated for 1 h at 37°C, and the released [$^{14}$C]oleic acid was extracted and measured as described elsewhere [24].

### 2.4. *Western blotting*

Rabbit anti-rat group II $PLA_2$ IgG was produced against $PLA_2$ released from thrombin-stimulated rat platelets, which was purified as previously described [25]. Immunoblotting with the anti-rat group II $PLA_2$ IgG was accomplished after SDS gel electrophoresis using a blotting detection kit (Amersham).

### 2.5. *cAMP assay*

After the incubation of SMCs, cAMP was extracted with 15% trichloroacetic acid from the cells. cAMP was measured with a cAMP assay system (Amersham).

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00145793/89/$3.50 

2.6. *RNA blotting*

Total cellular RNA was prepared by the method of Chomoczynski and Sacchi [26]. The RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde and transferred onto a nylon membrane by a standard method [27]. After immobilization of RNA by baking and UV cross-linking, hybridization was performed as described by Church and Gilbert [28]. Rat group II $PLA_2$ cDNA was labeled with [$^{32P}$-α]dCTP (New England Nuclear, 5000 Ci/mmol) using a random primer labeling system (Amersham), and used as a probe. After hybridization, the membrane was washed with 15 mM sodium citrate/1.5 mM NaCl/0.1% SDS at 65°C several times.

2.7. *Materials*

Human recombinant TNF and human recombinant IL-1β were purchased from Genzyme. LPS from *E. coli* was obtained from Pasel. Other chemicals were from Sigma.

## 3. RESULTS AND DISCUSSION

As reported for chondrocytes and synovial cells [4–7], IL-1 and TNF greatly enhanced the release of $PLA_2$ activity from SMCs (table 1). Moreover, we found that LPS also stimulated SMCs to secrete $PLA_2$ activity. To characterize this stimulation phenomenon further, we examined the effects of several agents on $PLA_2$ secretion from SMCs. The agents examined were as follows: forskolin, which stimulates adenylate cyclase; IBMX, which inhibits cAMP phosphodiesterase; dibutyryl cAMP, which is a membrane-permeable cAMP analogue; dibutyryl cGMP, which is a membrane-permeable cGMP analogue; which activates protein kinase C. As illustrated in table 1, the agents which increase intracellular cAMP concentrations (forskolin, IBMX and dibutyryl cAMP) enhanced the release of $PLA_2$ activity. On the other hand, dibutyryl cGMP and PMA had no effect on the secretion of $PLA_2$. The stimulation of $PLA_2$ secretion by forskolin or TNF was completely blocked by actinomycin D, an inhibitor of transcription, indicating that de novo RNA synthesis was necessary for enhancing the release of $PLA_2$.

Table 1

Phospholipase activity in the culture medium of SMCs incubated with various stimuli for 24 h

| Addition | Phospholipase activity |
|---|---|
| IL-1 (10 U/ml) | 12.6 |
| TNF (1000 U/ml) | 37.3 |
| LPS (100 ng/ml) | 33.4 |
| Forskolin (10 μM) | 17.3 |
| IBMX (0.5 mM) | 7.5 |
| Dibutyryl cAMP (1 mM) | 14.9 |
| Dibutyryl cGMP (1 mM) | 1.6 |
| PMA (100 nM) | 1.2 |
| Forskolin (10 μM plus actinomycin D (1 μM) | 0.8 |
| TNF (1000 U/ml) plus actinomycin D (1 μM) | 1.1 |
| Control | 1.0 |

Phospholipase activity is expressed as the relative change compared to the control sample. Data are the mean values from a representative experiment carried out in duplicate.


Fig.1. Elevation of group II $PLA_2$ mRNA levels in SMCs incubated with various stimuli. SMCs were incubated with vehicle (a), 10 μM forskolin plus 1 μM actinomycin D (b), 10 μM forskolin (c), 0.5 mM IBMX (d), 1 mM dibutyryl cAMP (e), 1 mM dibutyryl cGMP (f), 100 nM PMA (g), 1000 U/ml TNF (h) and 10 U/ml IL-1 (i) for 24 h. Total cellular RNA (15 μg/lane) was analyzed with RNA blotting as described in section 2.

To determine whether the stimulation of $PLA_2$ secretion occurred at the mRNA level, we performed RNA blot analyses. As shown in fig.1, levels of rat group II $PLA_2$ mRNA increased upon stimulation with IL-1, TNF, forskolin, IBMX, and dibutyryl cAMP. Actinomycin D inhibited the increase in the amount of $PLA_2$ mRNA, confirming that the elevation of $PLA_2$ mRNA level is a prerequisite to the stimulation of $PLA_2$ release. Since rat group I $PLA_2$ mRNA could not be detected in all cases (data not shown), the released $PLA_2$ from SMCs upon stimulation is a group II enzyme. Protein blotting analysis using anti-rat group II $PLA_2$ also showed that SMCs released group II $PLA_2$s in response to stimulations (fig.3).

The results described above suggest that cAMP acted as a second messenger for the stimulation of group II $PLA_2$ gene expression. In the forskolin-treated cells, intracellular cAMP concentrations transiently increased and remained much higher than in untreated cells even after 6 h of the incubation, as shown in fig.2. IL-1, TNF and LPS, however, did not change cAMP concentrations in SMCs, suggesting that the signal transduction mechanism evoked by these factors would be different from that involving cAMP. In order to elucidate whether TNF stimulation was mediated by a different mechanism (e.g. a different trans-acting factor) from that in cAMP stimulation, we examined the effect of addition of forskolin on the TNF stimulation. If forskolin and TNF stimulate the gene expression via the same mechanism, the output induced by forskolin plus TNF should not exceed the addition of the output induced by forskolin and TNF individually. However, as illustrated in table 2, the released $PLA_2$ activity induced by forskolin plus TNF was far above the sum of the individual effects. Forskolin (10 μM) increased the TNF-induced $PLA_2$ activity about ten times at all the concentrations of TNF. The synergistic effect of forskolin and TNF was also observed with Western blotting and Northern blotting (fig.3). IL-1 and LPS gave the same results as TNF (data not shown). These results indicated that IL-1-, TNF- and LPS-induced increases in the $PLA_2$ gene transcript were mediated by a different mechanism from that stimulated by the cAMP-elevating agents, and the two mechanisms acted synergistically to stimulate the mRNA synthesis.

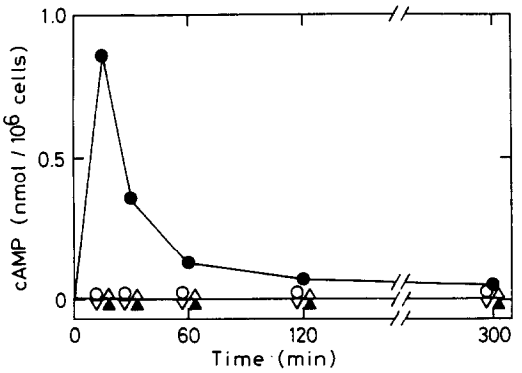


Fig.2. cAMP in the SMCs treated with 10 μM forskolin (●), 10 U/ml IL-1 (△), 1000 U/ml TNF (▲), 100 ng/ml LPS (▽) or vehicle (○). Data are mean values of triplicate experiments.

Recently, a study using human $PLA_2$ cDNA as a probe has shown that IL-1 stimulates $PLA_2$ mRNA synthesis in rabbit chondrocytes, although the specificity of the probe was not indicated [29]. In the present study, we clarified for the first time that the inflammatory mediators IL-1 and TNF, as well as bacterial LPS, stimulated the gene expression and secretion of group II $PLA_2$s but the rise in group I $PLA_2$ mRNA was not stimulated, by the use of rat group II $PLA_2$ cDNA and anti-group II $PLA_2$ IgG. Mammalian group II $PLA_2$s are found in some inflammatory regions [12–16] and have been shown to induce inflammatory responses [3]. These findings suggest the importance of the extracellular release of group II $PLA_2$ in promoting inflammatory processes.

Our findings suggest that group II $PLA_2$ plays an important role in the pathogenesis of vascular inflammatory processes such as vasculitis and atherosclerosis as well as the syndrome of septic shock. Recently, Pfeilschifter et al. also reported the release of $PLA_2$ activity from vascular SMCs [30]. IL-1 and TNF are produced by macrophages and endothelial cells, and LPS may be derived from infecting bacteria. Furthermore, the dramatic potentiation of the effects of IL-1, TNF and LPS by cAMP-elevating agents raises the possibility that some extracellular mediators which elevate the

Table 2

Phospholipase activity in the culture medium of SMCs incubated with TNF or TNF plus forskolin for 24 h

| TNF (U/ml) | Phospholipase activity | |
|---|---|---|
| | Without forskolin | With 10 μM forskolin |
| 0 | 1.0 | 6.3 |
| 1 | 1.3 | 9.8 |
| 10 | 3.1 | 43.2 |
| 100 | 9.6 | 115.7 |
| 1000 | 19.2 | 185.5 |

Phospholipase activity is expressed as the relative change compared to the control sample. Data are the mean values from a representative experiment carried out in duplicate.

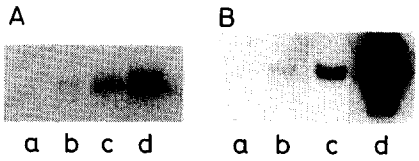


Fig.3. The synergistic effect of forskolin and TNF on the release of group II $PLA_2$s and the elevation of group II $PLA_2$ mRNA levels. SMCs were incubated with vehicle (a), 10 μM forskolin (b), 1000 U/ml TNF (c) or 10 μM forskolin plus 1000 U/ml TNF (d). The culture medium of the cells or total cellular RNA (15 μg/lane) was analyzed with Western blotting (A) or RNA blotting (B) as described in section 2.

intracellular cAMP level have a role in the progress of the above diseases by the combination with IL-1, TNF and LPS. In vascular systems, endothelial cells, SMCs and macrophages produce prostaglandins $E_2$ and $I_2$, which elevate the cAMP level in SMCs [31]. Such prostanoids may also have some roles in the progress of inflammation in addition to the regulation of contraction of vascular smooth muscle.

We are now studying the molecular mechanism of the regulation on group II $PLA_2$ gene transcription as well as the physiological role of $PLA_2$ in the progression of vascular inflammatory diseases.

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